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Layer-by-layer construction of the heparin/fibronectin coatings on titanium surface:stability and functionality

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Abstract

Layer-by-layer assembly as a versatile bottom-up nanofabrication technique has been widely used in the development of biomimetic materials with superior mechanical and biological properties. In this study, layer-by-layer assembled heparin/fibronectin biofunctional films were fabricated on titanium (Ti) surface to enhance the blood anticoagulation and accelerate the endothelialization simultaneously. The wettability and chemical changes of the assembled films were investigated by static water contact angle measurement and fourier transform infrared spectroscopy (FTIR). The morphology of modified Ti surfaces were observed using scanning electron microscopy (SEM). The real time assembly process was in-situ monitored by quartz crystal microbalance with dissipation (QCM-D). The stability of the films was evaluated by measuring the changes in wettability and the quantity of heparin and fibronectin on the surfaces. The anticoagulation properties of the films were quantitatively rated using Activated partial thromboplastin time (APTT) analysis. New peaks of hydroxyl and amino group were observed on the assembled Ti srufaces by FTIR. The contact angles varied among the films with different bilayer numbers, indicating the successful graft of the heparin and fibronectin layer-by-layer. QCM-D results showed that the frequency shift increased with the bilayer numbers, and the heparin and fibronectin could form multilayers. The assembly films were stable after incubation in PBS for 24 h based on the results of the contact angle measurement and the quantity of heparin and fibronectin analysis. APTT results suggested that the assembled films kept excellent antithrombotic properties. All these results revealed that the assembled heparin/fibronectin films with stabiltiy and anticoagulation property could be firmly formed on titanium surfaces. Our study further demonstrates that layer-by-layer assembly of heparin and fibronectin will provide a potential and effective tool for biomaterials surface modification.

PACS: R318.08

Keywords: Layer-by-layer assembly; Heparin; Fibronectin; QCM-D; Biomaterials

1. Introduction

Thrombosis on medical implants in contact with blood, such as heart valves, ventricular pump and vascular stents, etc., usually causes the failure of artificial devices and leads patients to the danger. Antithrombotic

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biomaterials have been of great interest in the development of artificial internal organs. The most popular method to obtain haemocompatibility is the modification of the materials themselves into antithrombotic materials. Up to data, different surface modifications techniques, e.g. thin film deposition with the proper composition and structure (DLC coating[1], ion sputtering[2], PIII&D[3], plasma[4], plasma induced micropatterning[5]), have been performed to improve the hemocompatibility of such kind of devices. Though minimizing thrombi and emboli generation and increasing lifetime of the devices[6], the lifelong anticoagulation therapy and long term thrombosis formation are inevitable.

Various antithrombotic molecules (covalent, ionic, physical adsorption) with specific chemical groups (e.g. -COOH, $-NH_2$, -OH) were immobilized onto the devices surface intend to further improve their blood-/bio-compatibility, Heparin and albumin are two common biomolecules which have been immobilized on biomaterials surface with excellent antithrombotic property. The covalent anchoring of RGD peptides on amine-reactive polymer ST-NH-PEG-PLA could significantly increase the adhesion and spreading of human osteoblasts [7]. The covalent immobilisation of tropoelastin on the plasma (C_2H_2) coated stainless steel surface was subsequently found to have improved biocompatibility by promoting endothelial cell attachment and proliferation relative to uncoated stainless steel controls [8]. M. Morra et al. [9] covalently linked collagen to the Ti surface, and the vivo experiments in rabbits showed that a significant increase of bone to implant contact. Bone ingrowth was observed on ColTi versus uncoated Ti fixtures. W. He et al [10] prepared nanoscale bioactive PEI-LN electrostatic layer-by-layer assembly coatings on silicon substrates. The PEI–LN multilayers were stable for at least seven days under physiological conditions (by ELISA) and significantly enhanced neuronal attachment. The layer-by-layer assembled chitosan/heparin coating on a coronary SS stent was proved to significantly promote re-endothelialization and was more safer for its better anticoagulation property compared with the bare metal stents [11]. Viewed from the present researches, most focus only on one aspect, anticoagulation or endothelialization. However, there are scare researches that considering the both sides simultaneously.

The layer-by-layer technique was introduced by Decher et al [12], the principle for this technique is the electrostatic interaction between a positive and a negative charged surfaces. The layer-by-layer technique is a promising approach for the construction of thin films containing biomacromolecules, such as polysaccharides [11,13], proteins [14,10] and enzymes [15]. Commercial pure titanium and its alloys have excellent resistance to corrosion and superior biological performance because of the passivating titanium oxide layer. They have been extensively used in orthopaedic [16] and dental [17] biomedical fields for manufacturing medical devices for their excellent biocompatibility, such as hip-joint replacement devices, dental implants, as well as heart-valves, however, it still faces with the problem of long-term thrombosis. In this study, layer-by-layer assembled HEP/FN multilayers were constructed on the Ti surfaces. HEP is a mixture of variably sulfated polysaccharide chains composed of repeating units of D-glucosamine and either L-iduronic or D-glucuronic acids (Fig.1a). It is the most widely chosen blood anticoagulant in clinic, and it has an incontrovertible effect on inhibiting thrombus formation by catalytically increasing the rate of antithrombin III (ATIII) and some other coagulating proteases. FN is a multifunctional extracellular glycoprotein found in plasma and the extracellular matrix. Thus it is now widely studied and used as a matrix for tissue engineering based on its critical role in cell attachment and migration processes (e.g. endothelial cells, fibroblast). Plasma FN consists of two similar subunits, which are 200–250 kDa molecular weight and are held together near the C terminus by two disulfide bonds. Each subunit is composed of homologous repeating structural modules (type I, II, and III). FN Type III modules are known to be involved in several interactions including heparin binding and cell binding via integrins (Fig.1b). We anticipate that this HEP/FN coating would be helpful to improve the biocompatibility of the Ti-based biomaterial devices [13].

The objective of this study was to fabricate and characterize the multilayers by the specific recognizing and binding between HEP and FN molecules, and to evaluate the stability and the anticoagulation property of the multilayers in vitro. We first investigate the synergetic effects of the layer-by-layer assembled HEP/FN layers on the improvements of the antithrombotic property and endothelialization of Ti surface.

2. Materials and Methods

2.1 Materials

Heparin sodium (HEP, >160 IU/mg) was supplied by Solarbio Corp., China and diluted to a concentration of 5 mg/ml with 50 mM 2- (N-Morpholino) ethanesulfonic acid Monohydrate (MES, purity: >99%), containing 1mM N-

Hydroxy-2,5-dioxopyrolidine-3-sulfonicacid sodium salt (NHS, purity: >99%) and 5 mM 1-ethyl-3-dimethylaminopropyl carbodiimide (EDC). Fibronectin (FN) was purchased from sigma-aldrich, USA and diluted to a concentration of 30μg/ml with phosphate-buffered saline (PBS) solution at pH 7.3. 3-aminopropyltriethoxysilane (APTE), Toluidine blue O (TB) and acid orange 7 were purchased from Sigma. All the other reagents were AR grade and used as received.

2.2 Preparation of the HEP/FN multilayer membranes

Commercial high pure titanium (Ti, Baoji, China) were used as the substrates for film growth. Firstly, Ti plates were cut into small squares (1 × 1 cm) and polished, then the plates were sonicated with acetone, ethanol, deionized water and finally dried at room temperature (RT). Fig 1c shows the scheme of the formation of HEP/FN multilayers, the cleaned Ti plates were immersed in a 1 M NaOH solution at 70 °C for 24 h (sample was labeled as TiOH), then rinsed thoroughly with deionized water and subsequently immersed in a 2 % (v/v) ethanol solution of APTE for 10 h at 37 °C with gentle shaking. After reaction, the carriers were washed with the same solvents and kept in a 120 °C oven for 10 h to enhance the binding of APTE with the carrier (sample was labeled as TiOH-APTE) [18]. The aminolyzed substrates were then dipped in the 5 mg/ml heparin sodium salt MES solution for 15 min and subsequently rinsed with PBS. The heparinized substrates were then placed into the solution made with PBS and 30 μg/ml FN for 15 min, followed by the same rinsing procedures. Several bilayers of HEP and FN were prepared by repeating the depositon process mentioned above, to produce a stable supramolecular complex film, HEP was the outmost layer for all the samples. Finally, the samples were dried for 48 h at RT [11,19].

2.3 Quartz crystal measurement of the HEP/FN multilayer membranes

A Q-Sense E4 system (Q-Sense AB, Sweden) was used to monitor the multilayer buildup in real time. In situ dissipative quartz crystal measurement analysis mode was employed as reported [22]. A Ti-coated quartz crystal was initially treated with UV irradiation for 10 min for surface cleaning and sterilizing, then settled in the measurement chamber and ethanol was injected as a buffer for equilibrium. A 2% ethanol solution of APTE was injected at 50 μl/min continuously until the adsorption reached equilibrium and stabilized for 24 h, then rinsed with ethanol and PBS. The trace of APTE adsorption was selected as baseline. Subsequently, HEP solution was injected until no variation appeared in the adsorption curves. PBS was then pumped in again and FN solution was injected thereafter at the same speed for the next equilibrium. HEP and FN were then alternately pumped into the chamber for the buildup of multilayers on the quartz crystal surface. The 15, 25, 35 and 45 MHz overtones were selected to extract resonance frequencies [23]. Frequency shift vs. time (F–t) curves were recorded to monitor the assembly and stability of the adsorbed HEP/FN multilayer membranes. All measurements were performed at 37 °C.

2.4 Surface characterization of hydroxy and APTES Ti

The characteristic absorption peaks of the hydroxyl groups and amino groups of unmodified and modified Ti were detected using a Fourier transform infrared spectrometer (FTIR, NICOLET 5700, USA). The surface densities of amino groups wes determined from the uptake of an acid dye. Amino groups on the membrane can form complexes with acid orange 7 at pH 3, and then the complex dye was desorbed with 1 mM NaOH. The absorbance of the supernatant at 485 nm was then measured. For morphological observation, the modified and original Ti samples were observed by scanning electron microscopy (SEM, QUANTA 200, FEI, Netherland).

2.5 Stability test of the multilayers

The samples were immered into a 24-well culture plate containing PBS for 24 h to examine the stability of the multilayers,. Then the modified samples were removed from the solution and analyzed by the contact angle measurements, surface density of HEP and FN before and after contact with PBS, respectively. Three samples were used for each test.

2.5.1 Contact angle

The contact angle analysis with double-distilled water in this study was performed using a contact angle goniometer (JY-82, Tianjin, China). More three different points were measured for each sample in order to get statistical averages. All measurements were performed at ambient temperature.

2.5.2 Surface density of heparin

The sulfonic groups on the surface can form complexes with toluidine blue O (TB) dye. To determine the surface density of immobilized heparin [24], the modified Ti samples with HEP and FN were immersed in 1 ml 50 mg/L TB for 2 h, then n-hexane (3 ml) was added and the mixture was shaken well to ensure uniformity of the dye. After removing the samples from the solution, the aqueous layers of the solution were sampled. The absorbance at 631 nm was then measured by UV spectrophotometry (BIO-TEK instruments, USA) and the amount of immobilized heparin was calculated from the calibration curve of free heparin. The residual ratio of immobilized heparin were calculated as follows:

$$\text{Residual ratio (\%)} = \frac{R0\text{-}Rt}{R0} \times 100\%$$

where R0 and Rt are the surface density of immobilized heparin on the multilayer membranes before and after contact with PBS, respectively.

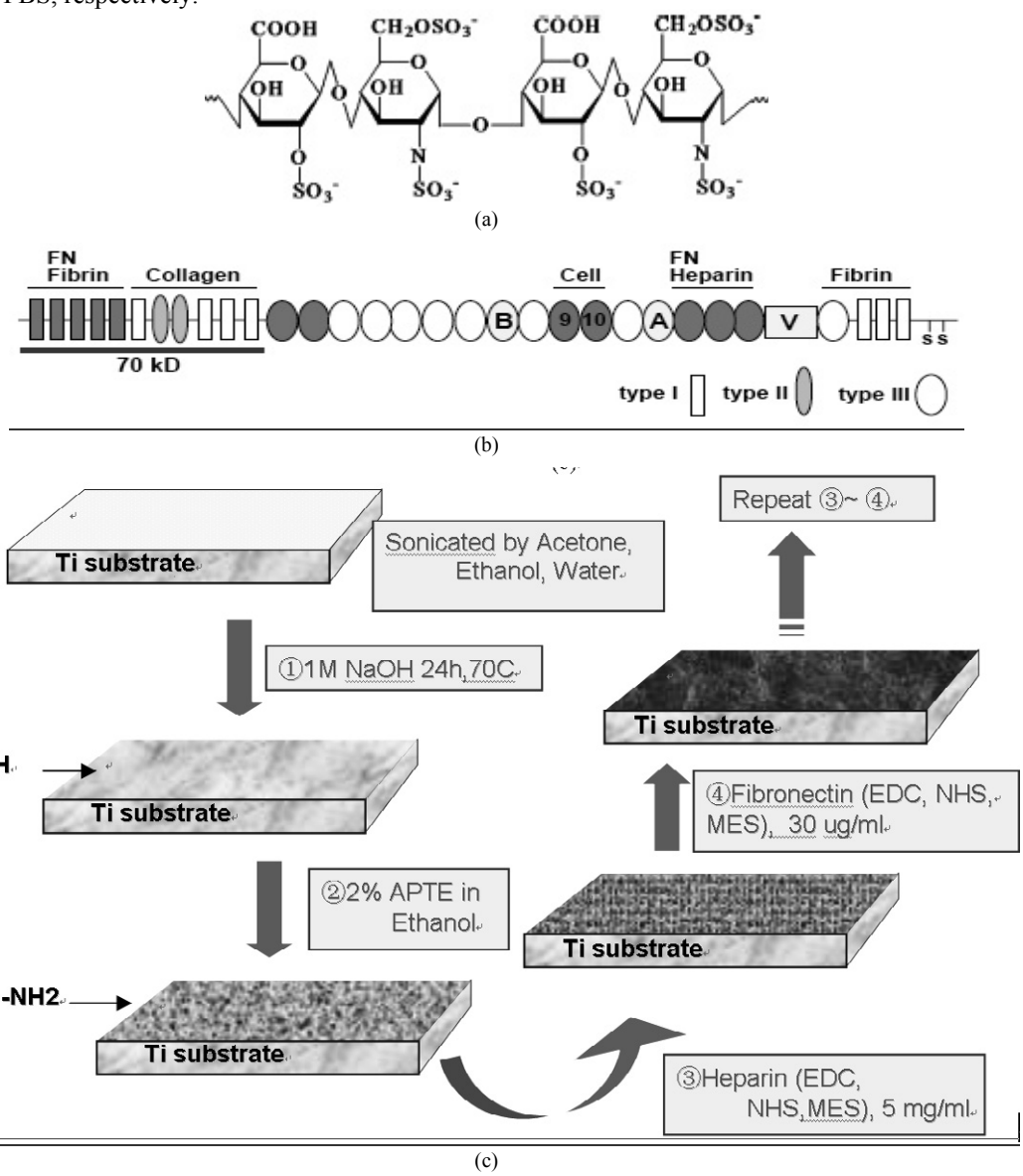

Fig.1. The chemical scheme of : a) the structure of HEP [20]; b) the structure of FN [21]; c)the construction of HEP/FN multilayers

2.5.3 Surface density of fibronectin

The amount of FN was determined by enzyme-linked immunosorbent assay (ELISA) as following: HEP and FN modified Ti samples were blocked with sheep serum (1/100 dilution in PBS) for 30 min, and then washed with PBS for three times and incubated with mouse anti-human FN antibody (1/250 dilution in PBS) for 1 h. Subsequently, rinsed with PBS again and incubated with horseradish peroxidase labelled goat anti-mouse antibody (1/100 dilution in PBS) for another 1 h. Finally, washed and colored with TMB reagent. The absorbance was obtained at 450 nm.

2.6 Antithrombotic properties test of the multilayers-APTT test

Anticoagulant human whole blood (30 ml) from a healthy volunteer was supplied by Chengdu Blood Center, and then the blood was centrifuged at 3000 rpm for 15 min to separate the blood corpuscles. The platelet-poor plasma (PPP) obtained was used for activated partial thromboplastin time (APTT) test. The Ti samples were put into a 24-well culture plate and added 500 μl PPP per well and incubated for 15 min at 37 °C. 100 μl of actin activated thromboplastin reagent (Huachen, Shanghai, China) was put in a glass tube, and the tube was incubated at 37 °C for 1 min. Then 100 μl of PPP solution from sample well was added at 37 °C, it was incubated for 3 min, and then 100 μl of 30 mM CaCl2 solution was added. The clotting time of the plasma solution was recorded at the first sign of fibrin formation with a hook.

2.7 Statistical analysis

The data were reported as mean±standard deviation. The statistical analysis between different groups were performed using student's t-test. The probabilities of $P< 0.05$ were considered as significant difference.

3. Results and discussion

3.1 Surface characterization—hydroxyl and APTES

Fig.2 shows the FTIR spectra of the bare Ti and modified Ti samples. Compared with the orginal Ti surface (Fig.2a), the TiOH surface (Fig.2b) showed a new peak at 3400 cm^{-1} approximate to the -OH group. The APTE grafted surface (Fig.2c) had new peaks at 2920 cm^{-1} approximate to -CH2 and -CH3, indicating APTE derived on the surface.

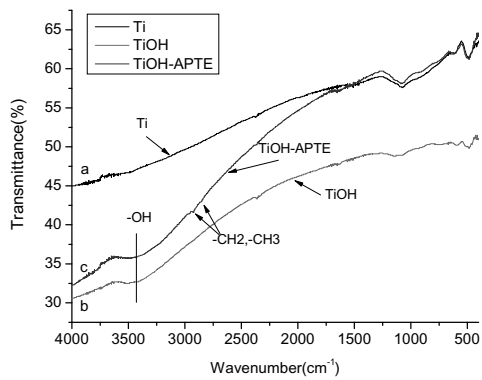

Fig.2. The ATR-FTIR spectra of: (a) Ti; (b) TiOH; and (c) TiOH-APTE.

The peak of –NH2 at 3200 cm^{-1} was not sensitive to FTIR and can hardly be found in the FTIR spectrum, however, the -NH2 group could be detected by complexation with the anionic dye, acid orange 7 (5×10^{-4} M, pH 3), as shown in Fig.3. A significant increase of amino density was seen on TiOH-APTE surface compared to Ti and TiOH surfaces. Si-O stretching peak at 1100 cm^{-1} was superposed as it also presented on the film sample. Fig.4 depicts the SEM images of Ti, activated by NaOH (TiOH) and TiOH grafted with APTE (TiOH-APTE). Compared with Ti surface (Fig. 4a) and TiOH surface (Fig.4b), TiOH-APTE surface (Fig.4c) had the largest roughness due to the chain structure of APTE. TiOH surface just had -OH grafted on it, while APTE could be crosslinked with each other and increased the surface roughness. These results indicated the existence of APTE on the modified Ti surface.

3.2 Monitoring of the assembly of HEP and FN by QCM-D

QCM-D was applied to monitor the assembly process of HEP and FN on Ti coated quartz crystal surface in real time. Frequency shift vs. time curves (Fig.5) clearly demonstrated the layer by layer buildup process of the HEP/FN coating. Upon injection of the HEP or the FN solution and alternatively, the crystal frequency decreased, indicating the adsorption of the HEP or the FN molecules onto the crystal surface. The formation of the ladder-like QCM-D traces suggested that the layer-by-layer structure of the HEP/FN membranes were constructed on the Ti surface. No frequency shift occurred after the sample rinsed with PBS, which indicated that the adsorpted of HEP/FN multilayers were stable.

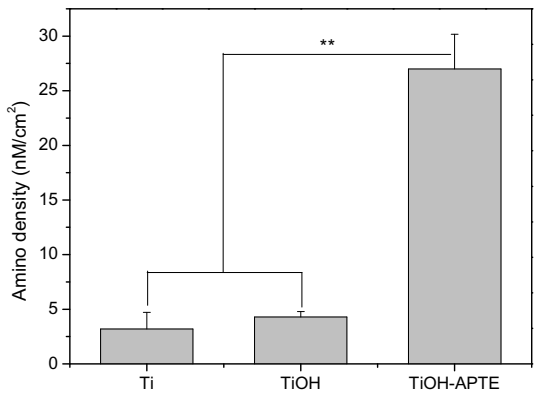

Fig.3. Amino density on the surface of Ti, TiOH and TiOH-APTE. Error bars represent means±SD. n =3. **P<0.05 (compared to control Ti and TiOH).

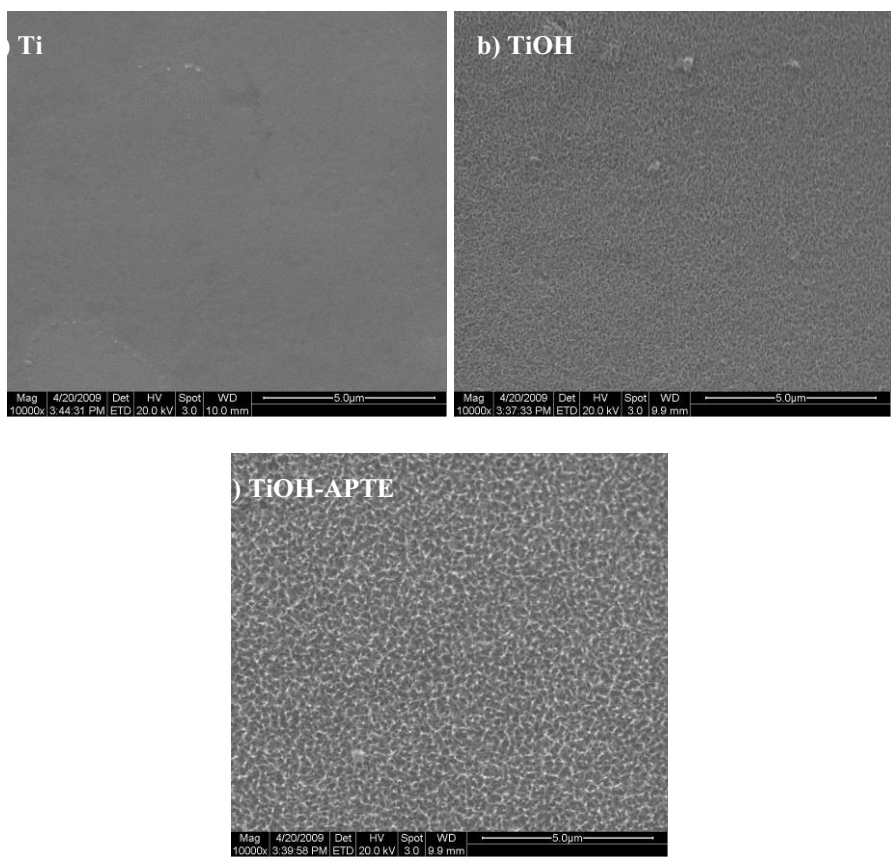

Fig.4. SEM images of :(a) Ti; (b) TiOH; and (c) TiOH-APTE. 10000×

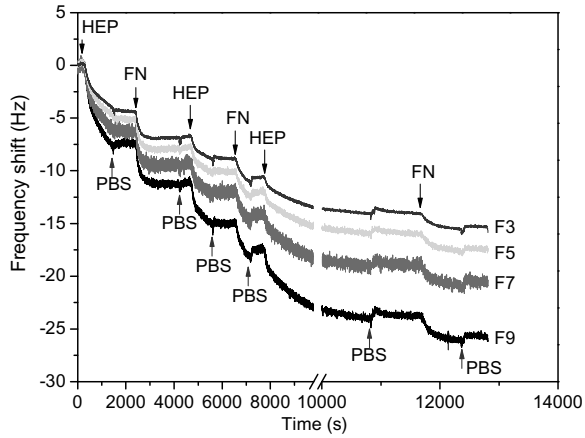

Fig.5. Frequency (F) as a function of time during the buildup of HEP/FN multilayers onto a Ti coated quartz crystal from 5 mg/ml HEP and 30 μg/ml FN solutions. The down arrows indicate the injections of HEP or FN solution. The up arrows correspond to the rinse of PBS. Data are shown in four overtones: F3, F5, F7 and F9.

HEP was found to rapidly adsorbed on the Ti surface evidenced by the sharp frequency shift. It reached equilibrium slowly and coincided with the injection, whereas FN reached equilibrium quickly. This phenomenon might be caused by the high concentration of HEP (5 mg/ml) and low concentration of FN (30 μg/ml). HEP molecules have a competitive adsorption abilty and it would be rearranged on the Ti surface during the adsorption. Similar phenomenon was not observed on the FN molecules.

3.3 Contact angle

Fig.6 shows the contact angles of water droplets on the HEP/FN multilayers before- and after-PBS immersion. HEP was known to be more hydrophilic than FN. The hydrophilic/hydrophobic characteristic of the multilayer films were expected to change due to the physical and chemical environment variation of the outermost layer. When the outermost layer was covered by FN, the water contact angle was higher than that when it was covered by HEP. The serrated curves reveal that the of HEP and FN membranes were distributed layer-by-layer on the Ti surface. The contact angles were decreased evidently for the Ti samples immersed in PBS for 24 h, however, no significant changes were observed for the multilayer films, indicating the multilayers were stable.

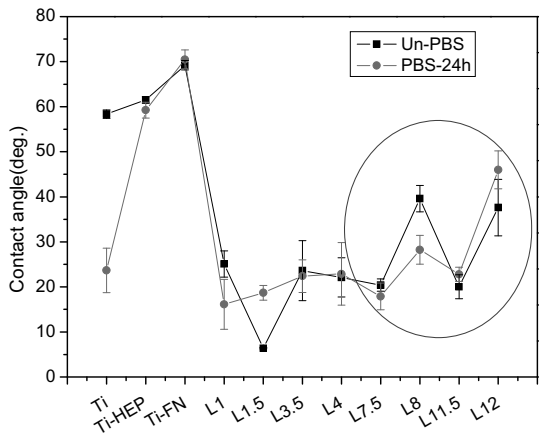

Fig.6. Water contact angle as a function of the number of coating layers. The integer layer numbers correspond to FN as the outermost layer; the other layer numbers correspond to HEP as the outermost layer.

3.4 Quantitative characterization of HEP and FN

Quantitative characterization of HEP and FN before- and after- contact with PBS were performed to further investigate the stability of the multilayer films. The surface densities of HEP on the multilayers were showed in Fig.7. It can be seen clearly that the surface density of HEP increased monotonously with the layer numbers. The

amount of HEP on all the multilayered samples was larger than those on Ti surface. But it was decreased after the samples were immersed in PBS for 24 h. Almost no HEP were left for the layer 1.5 compared with that on Ti surface, indicating the complete release of HEP and the instability of HEP/FN layers. Nevertheless, the assembly layers became stable and less HEP released when the layer numbers increased, When the assembled layers were L11.5, the residual percentage of HEP on multilayers reached to about 90%. Y. Byun et al. [25] studied a styrene/p-amino styrene random co-polymer coupled with poly (ethylene oxide) (PEO) to bind HEP, and the amount of immobilized HEP was 4.9 μg/cm^2. The result here indicated that the amount of HEP on our multilayer films after immersion in PBS for 24 h was larger than that reported in the literature.

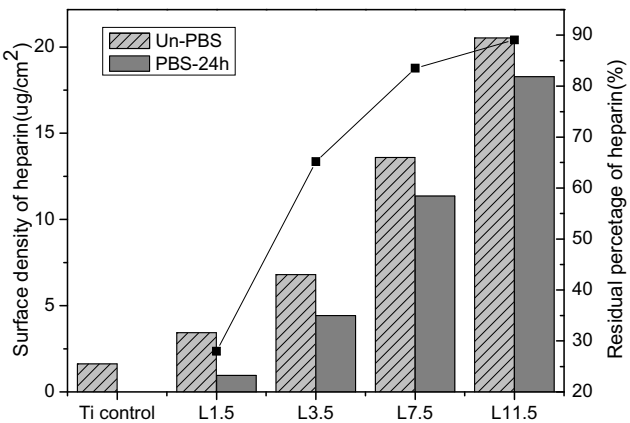

Fig.7. The surface density and residual percentage of heparin on multilayers before- and after- contact with PBS for 24h. The outmost layer is HEP. Ti is as the control sample.

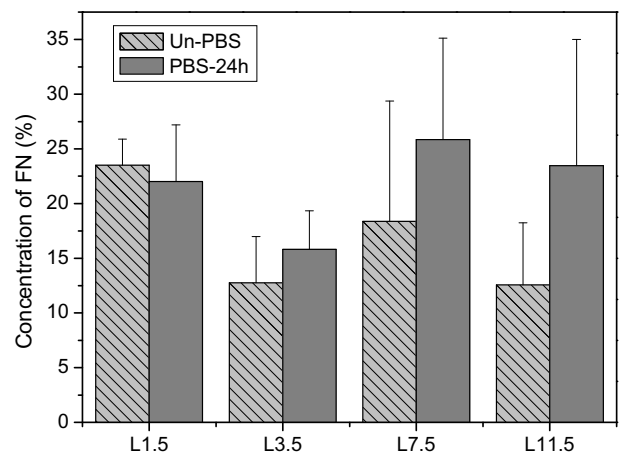

Fig.8. The concentration of FN on multilayers before- and after- immersion in PBS for 24h. The outmost layer is HEP.

The amount of FN was determined by ELISA. Fig.8 shows the concentration of FN on multilayer films before- and after- immersion in PBS for 24h. No obvious difference was seen for all the samples before- and after- contact with PBS. The samples (L3.5, L7.5 and L11.5) after contacting with PBS for 24 h even had larger FN concentration than that with no treatment. The reason for this may be due to the concentration of FN for the samples that un-contacted with PBS just referred to the FN on the surface of the multilayers, but when the samples were immersed in PBS, the multilayer films would be hydrolysis and became less compact. Note that both HEP and FN would release during the immersion from Fig.7 and Fig.8, and more FN molecules could be emerged because of the release of more HEP and FN. For the sample L1.5 with the structure HEP-FN-HEP, a decrease of FN concentration was observed due to the only one layer of FN on this sample, and no further FN exposure during the immersion.

3.5 In vitro haemocompatibility of the HEP/FN multilayer films

HEP had already been proved to be efficient in the anticoagulation of human blood. APTT results could reflect the antithrombotic properties of the HEP/FN multilayer films. The normal ranges of APTT for healthy human

plasma was regarded to be 34±7 s. Fig.9 reveals that the APTT of untreated Ti slightly decreased compared with that of plasma. It was found that the APTT values of all the HEP/FN samples before PBS immersion were significantly prolonged (> 180 s), and the values extended by almost 6 times than that of the plasma. These values became smaller after the samples were contacted with PBS for 24 h, but still longer than that of the plasma and the positive control Ti. The APTT values of the multilayered samples were also found to be extended with the increase of the number of HEP/FN layers.

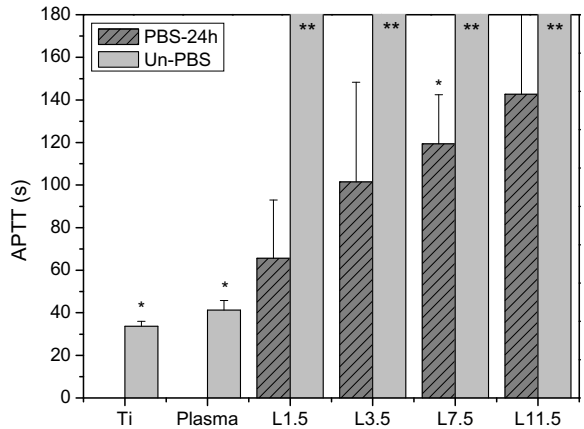

Fig.9. APTT of HEP/FN multilayer films with 1.5, 3.5, 7.5 and 11.5 layers, respectively; Ti was used as the control (*p < 0.05, ** data exceeded 180 s).

M.C. Yang et al. [19] studied the blood compatibility of polyacrylonitrile membrane with immobilized chitosan-heparin conjugate, they found that APTT was related to the thrombogenesis initiated by the adsorption of protein (fibrinogen), and the protein adsorption decreased while the APTT increased, indicating that by immobilizing HEP onto surface, blood coagulation could be reduced. The antithrombotic properties of the HEP/FN films are mainly attributed to the heparin. Our results showed that the multilayered samples still kept excellent anticoagulation properties after immersion in PBS for 24 h.

4. Conclusion

In this study, hydroxyl was produced on Ti surface by NaOH, and APTE was successfully grafted on TiOH surfaces. The HEP/FN multilayered films were constructed by layer-by-layer assembly technique. This kind of HEP/FN assembled film was found to be stable under simulated physical condition for 24 h. Moreover, the HEP/FN multilayered films kept anticoagulation properties and displayed better heamocompatibility. The HEP/FN multilayers provides a possible resolution for the rapid endothelialization and anticoagulation simultaneously, thus may have a great potential for future application. Ongoing efforts are focused on the structure analysis, bloodcomptibility and endothelial cells compatibility of the multilayered films

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